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Induction of free radicals in hepatocytes, mitochondria and microsomes of rats by ochratoxin A and its analogs

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Abstract

Oxidative damage may be one of the manifestations of cellular damage in the toxicity of ochratoxin A (OA). OA; its three natural analogs, OB, OC and Oα; and three synthetic analogs, the ethyl amide of OA (OE-OA), *O*-methylated OA (OM-OA), and the lactone-opened OA (OP-OA) were used to study free radical generation in hepatocytes, mitochondria and microsomes from rats. Electron paramagnetic resonance spectroscopy (EPR) using α-(4-pyridyl-1-oxide)-*N*-*tert*-butyl nitrone (4-POBN) as a spin trapping agent showed an enhanced free radical generation due to the addition of NADPH to the microsomes. An EPR signal was not observed in the mitochondria and hepatocyte samples when they were treated with a variety of agents. Addition of OM-OA together with NADPH and Fe^{3+} to the microsomes resulted in a strong EPR signal compared with the other analogs, whereas the signal could be quenched by the addition of catalase. OM-OA does not have a dissociable phenolate group and does not chelate Fe^{3+}. The spin adduct hyperfine splitting constants indicated the presence of α-hydroxyethyl radicals resulting from generated hydroxyl radicals, which were trapped by 4-POBN. The results also suggested that the production of hydroxyl radicals by OA does not require a dissociable phenolate group or the prior formation of an OA-Fe complex. © 1997 Published by Elsevier Science B.V.

Keywords: Ochratoxin; Free radical; Electron paramagnetic resonance; Hepatocyte; Mitochondrion; Microsome

1. Introduction

Ochratoxin A (OA) is a mycotoxin that has a widespread occurrence in food and feed. It has been shown to be nephrotoxic, hepatotoxic, teratogenic, carcinogenic and immunosuppressive. The direct biochemical evidence for some of these toxicological effects still remains to be clarified. In recent years it has been demonstrated that lipid peroxidation is one of the manifestations of cellular damage in the toxicity of some mycotoxins including OA. However, the importance of free radical generation and lipid peroxidation as a direct cause of the resulting toxicities has not been established. Rahimtula et al. [1] showed that OA, when added to rat liver microsomes, enhanced the rate of NADPH or ascorbate-dependent lipid peroxidation as measured by malondialdehyde formation. In vivo administration of OA to rats also

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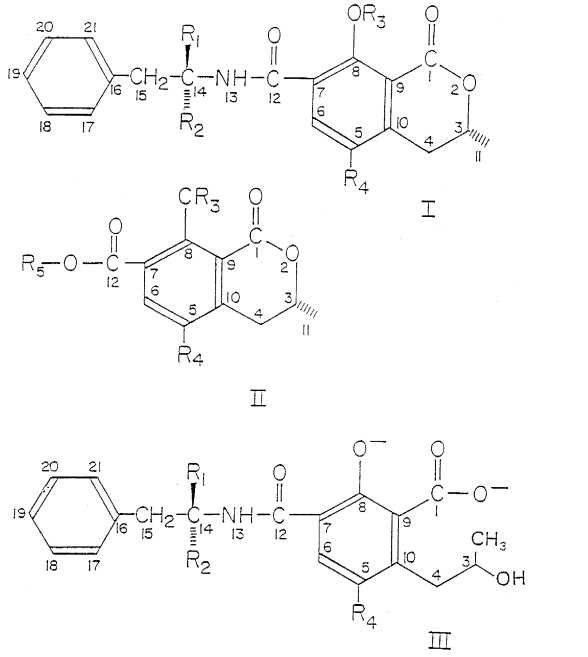
resulted in enhanced lipid peroxidation. It has also been reported that some of the toxic effects of OA in vitro and in vivo can be ameliorated or counteracted by antioxidants such as vitamin C and E [2–5]. No EPR spin trapping study, to the best of authors' knowledge, has been published on the effects that OA has on microsomes, mitochondria or hepatocytes. Hasinoff et al. [6], however, used isolated flavoprotein NADPH-cytochrome-*P*-450 reductase to study free radical generation by an Fe^{3+} complex of OA. In the current study, several analogs of OA were evaluated to determine if there was a structural requirement for the production of hydroxyl radicals, especially if there was a relationship between the iron binding capacity of these analogs and their ability to produce free radicals as proposed by Rahimtula et al. [1]. The analogs studied were OA; its three natural analogs, OB, OC and Oα; and three synthetic analogs, the ethylamide of OA (OE-OA), *O*-methylated OA (OM-OA), and the lactone-opened OA (OP-OA). Recently, Hoehler et al. [7] found that OA induces hydroxyl radical production in bacteria as evidenced by EPR spectroscopy and that OA enhances the permeability of the cellular membrane to Ca^{2+} in this model system. Therefore, in the present study, the induction of free radicals in microsomes, mitochondria and hepatocytes from rats by OA and several of its analogs was investigated.



Analog	Structure	R1	R2	R3	R4	R5
OA	I	COOH	H	H	Cl	-
OB	I	COOH	H	H	H	-
OC	I	$COOCH_2CH_3$	H	H	Cl	-
OE-OA	I	$CONHCH_2CH_3$	H	H	Cl	-
OM-OA	I	COOH	H	CH_3	Cl	-
Oα	II	-	-	H	Cl	OH
OP-OA	III	COOH	H	-	Cl	-

Fig. 1. Structures of ochratoxin A (OA) and its analogs.

2. Materials and methods

2.1. Ochratoxins and chemicals

OA, OB and Oα were isolated from a culture of *Aspergillus ochraceus* NRRL 3174 [8]. OE-OA, OM-OA and OP-OA were synthesized as described previously [8,9]; OC was synthesized according to van der Merwe et al. [10,11] (see Fig. 1). The toxins were more than 99% pure and did not contain more than 1% of other forms of OA. The toxins were dissolved in ethanol. The spin trapping agent, 4-POBN, as well as *dl*-α-tocopheryl acetate, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), the Ca ionophore A23187, digitonin, calcium chloride, ferrous sulfate, anhydrous ferric chloride, superoxide dismutase (SOD), catalase (CAT), *N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid) (Hepes), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA) and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest grade commercially available. The Fe^{3+} complexes of the different ochratoxins were pre-formed by adding $FeCl_3$ in ethanol to the ochratoxin also in ethanol as described previously [6].

2.2. Preparation of rat liver microsomes

Liver microsomes were prepared from adult female Sprague-Dawley rats according to Palozza et al. [12]. In brief, tissues were homogenized with 5 volumes of ice-cold 0.25 M sucrose containing 5 mM Hepes, 0.5 mM EDTA, pH 7.5 in a Polytron homogenizer (Polytron, Kinematica GmbH, Luzern, Switzer-

land). Microsomal vesicles were isolated by removal of the nuclear fraction at $8000 \times g$ for 10 min and removal of the mitochondrial fraction at $18\,000 \times g$ for 10 min. The microsomal fraction was sedimented at $105\,000 \times g$ for 60 min, washed once with 0.15 M KCl and collected again at $105\,000 \times g$ for 30 min. The membranes were suspended in 0.1 M potassium phosphate buffer, pH 7.5, the protein concentration was determined according to Bradford [13] using BSA as a standard and adjusted to 4 mg/ml. The livers of 10 rats were pooled for these preparations and the samples were stored at −80°C.

2.3. Preparation of rat liver mitochondria

Liver mitochondria were isolated from adult female Sprague-Dawley rats which were fasted overnight according to the method of Johnson and Lardy [14] with modifications according to Kennedy et al. [15]. In brief, the livers were minced in a petri dish on ice, homogenized in 30 ml ice-cold isolation solution (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 0.15% BSA) with 10 strokes of the Polytron homogenizer (Polytron, Kinematica GmbH, Luzern, Switzerland) and centrifuged at $600 \times g$ for 10 min. Mitochondria were washed in the same medium without EDTA, EGTA and BSA and centrifuged again at $600 \times g$ for 10 min. The mitochondria were pelleted at $9500 \times g$ for 5 min to minimize peroxisomal contamination. The pellets were resuspended in the isolation solution and the protein concentration was determined [13] and adjusted to 4 mg/ml.

2.4. Preparation of rat hepatocytes

Hepatocytes from female Sprague-Dawley rats weighing 100–150 g were isolated by a collagenase perfusion technique [16] and suspended in Dulbecco's modified Eagle's medium containing 10% dilapidated fetal calf serum and 2% fatty acid-free albumin as described previously [17]. The final preparation of the hepatocytes was counted in the presence of 0.04% trypan blue using a hemocytometer and diluted to $20 \pm 2 \times 10^6$ cells/ml. Hepatocytes were placed in a horizontal shaker at 37°C and used for EPR measurements within a few hours.

2.5. EPR spin trapping

The samples (microsomes, mitochondria or hepatocytes) were incubated for up to 16 scans (1 scan = 70 s) at 37°C with 100 mM 4-POBN. All the reaction mixtures contained 2% v/v ethanol. The spectra were measured with a Varian Associates Model E-12 EPR spectrometer operating at 9.02 GHz with 100 kHz modulation which was interfaced with a Nicolet Instruments Model 1180 computer and Model 2090 transient recorder. The instrument settings were: microwave power of 20 mW; modulation amplitude of 1.6 G, and scan range of 100 G. The sample temperature was controlled at 37°C by a Bruker Model ER-4111 temperature controller. All EPR spectra were recorded in identical 0.8 mm (i.d.) glass capillary tubes from Kimble Products. Spectra shown in each figure were carried out on the same day and are representative of at least three independent experiments.

3. Results

The spin adduct hyperfine splitting constants ($\pm$S.D.) of the 6-line signals using 4-POBN as a spin trapping agent were 15.4 G for a_N and 2.5 G for a_H ($\pm$0.03 G). This species, as indicated in Section 4 [18–20], is characteristic of the 4-POBN-α-hydroxyethyl spin adduct and is produced by the reaction of the hydroxyl radical ($^{\cdot}OH$) with ethanol. All of the reaction rates were linear for at least 10 min (8 scans at 70 s each), which was also similar to the results obtained by Rashba-Step et al. [21] when hepatic microsomes were incubated with NADH or NADPH.

The addition of 2.5 mM OA, 2.5 mM Fe^{3+} or OA and Fe^{3+} in combination to the microsomes resulted only in very weak EPR signals, whereas a strong EPR signal could be obtained by the addition of 1 mM NADPH (Fig. 2). OA in combination with NADPH increased the signal by 25% (Fig. 2C), while Fe^{3+} in combination with NADPH increased it by 150% (Fig. 2B). The magnitude of the signal obtained with a combination of OA, Fe^{3+} and NADPH (Fig. 2A) was slightly less than that obtained with NADPH and Fe^{3+}. These results suggest that both OA and Fe^{3+} in the presence of NADPH stimulated the production of hydroxyl radicals by the micro-

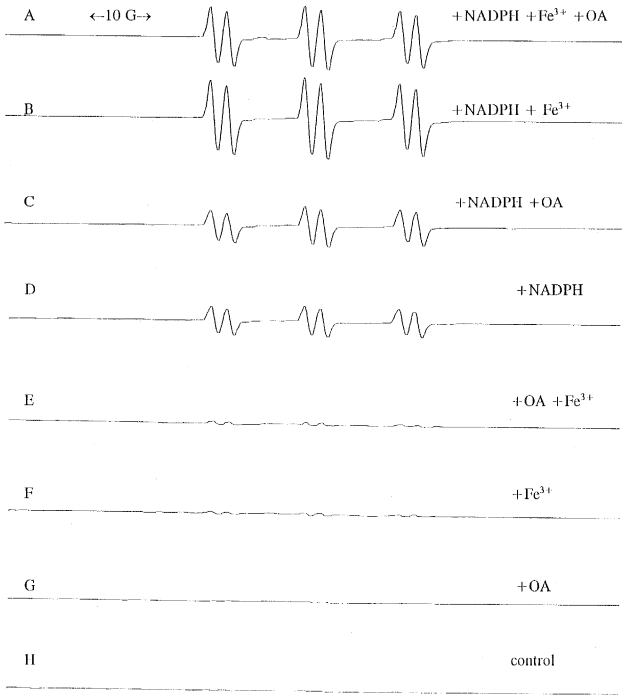


Fig. 2. EPR spectra of 4-POBN radical adducts following the incubation of rat liver microsomes with NADPH, OA, Fe^{3+}. Rat liver microsomes were isolated and incubated as described under Section 2. The spectra were recorded at 37°C immediately after adding 2% (v/v) ethanol and 100 mM 4-POBN, and were accumulated 8×. In addition, the samples contained: (A) 1 mM NADPH, 2.5 mM OA, 2.5 mM Fe^{3+}; (B) 1 mM NADPH, 2.5 mM Fe^{3+}; (C) 1 mM NADPH, 2.5 mM OA; (D) 1 mM NADPH; (E) 2.5 mM OA, 2.5 mM Fe^{3+}; (F) 2.5 mM Fe^{3+}; (G) 2.5 mM OA; (H) control, no additions to microsomes containing ethanol and 4-POBN.

somes with the effect being much greater in the presence of Fe^{3+} than in the presence of OA and that the effects of both OA and Fe^{3+} when present together were not additive. In another study, the effect of preincubating Fe^{3+} with OA to form an OA-Fe^{3+} complex was compared to results obtained without prior formation of this complex (Fig. 3). The addition of the OA-Fe^{3+} complex to the microsomal-NADPH preparation (Fig. 3B) yielded a signal that was slightly less in amplitude relative to that obtained with NADPH and Fe^{3+} (Fig. 3C). The addition of both OA and Fe^{3+} without the prior formation of an OA-Fe^{3+} complex did not affect the magnitude of the EPR signal (Fig. 3A). These results therefore suggest that the magnitude of the EPR signal is not dependent upon prior formation of an OA-Fe^{3+} complex. Hasinoff et al. [6], in contrast to results obtained in this study, reported that very little 6-line spectra were observed when OA was omitted from the complete system (NADPH cytochrome-*P*-450 reductase plus NADPH), that either Fe^{3+} or OA alone increased the signal and presumably to a similar degree, and that both OA and Fe^{3+} together as a complex produced a very distinct 6-line spectrum. They concluded that both Fe^{3+} and OA were required to induce a maximal signal and that complexation of Fe^{3+} by OA was necessary for hydroxyl radical production. The data from Figs. 2 and 3 do not support these conclusions.

Different analogs of OA were incubated in the presence of microsomes, NADPH and Fe^{3+} (Fig. 4). The OA analogs did not alter the EPR signal significantly, except the *O*-methylated analog OM-OA caused a distinct two-fold increase of the signal (Fig. 4B vs. 4H). In comparison with OA (Fig. 4G), the OM-OA signal was 2.3 times stronger. The signal due to OM-OA as presented in Fig. 5 (B) was even stronger (2.8 times) than that of OA (Fig. 5D). To investigate the sensitivity of this system to exogenous catalase, which catalyzes the decomposition of H_2O_2 to O_2, the enzyme was added to both the OA and OM-OA treated microsomes (Fig. 5A,C). The addition of catalase greatly reduced the EPR signals, suggesting that H_2O_2 and possibly other reactive oxygen species are involved in this free radical generating process. The addition of superoxide dismutase (0.1 mg/ml) to the microsomes containing NADPH,

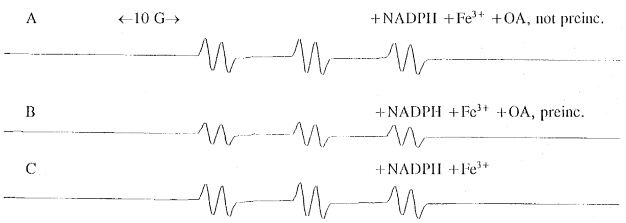


Fig. 3. EPR spectra of 4-POBN radical adducts following the incubation of rat liver microsomes with NADPH, OA and Fe^{3+}. Rat liver microsomes were isolated and incubated as described under Section 2. The spectra were recorded at 37°C immediately after adding 1 mM NADPH, 2% (v/v) ethanol and 100 mM 4-POBN, and were accumulated 8×. In addition, the samples contained: (A) 0.83 mM Fe^{3+}, 2.5 mM OA, not preincubated; (B) 0.83 mM Fe^{3+}, 2.5 mM OA, preincubated for 15 min to preform the Fe^{3+}-OA complex; (C) 0.83 mM Fe^{3+}.

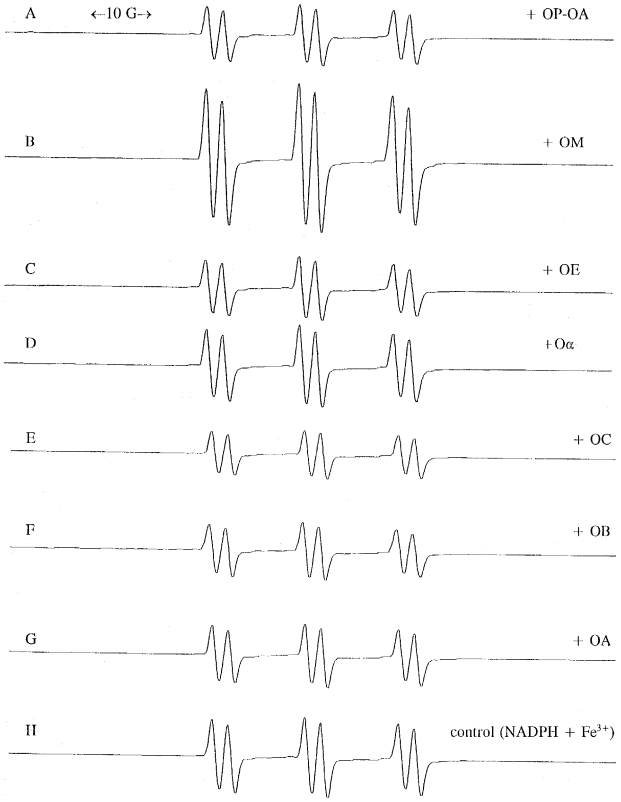


Fig. 4. EPR spectra of 4-POBN radical adducts following the incubation of rat liver microsomes with NADPH, Fe^{3+} and the OA analogs. Rat liver microsomes were isolated and incubated as described under Section 2. The spectra were recorded at 37°C immediately after adding 1 mM NADPH, 2.5 mM Fe^{3+}, 2% (v/v) ethanol and 100 mM 4-POBN, and were accumulated 8×. In addition, the samples contained: (A) 2.5 mM OP-OA; (B) 2.5 mM OM-OA; (C) 2.5 mM OE-OA; (D) 2.5 mM Oα; (E) 2.5 mM OC; (F) 2.5 mM OB; (G) 2.5 mM OA; (H) control.

Fe^{3+} and either OA or OM-OA increased the signal slightly (7%, data not shown).

The spectra shown in Fig. 6 were obtained by the addition of the different agents to the microsome storage solution (0.1 M K_2HPO_4, pH 7.5) and contained neither microsomes nor NADPH. A weak signal was produced by H_2O_2 and Fe^{3+} (Fig. 6E). OA (Fig. 6D) or OM-OA (Fig. 6C) added in combination with H_2O_2 did not generate an EPR signal. When the samples were treated with OA, H_2O_2 and Fe^{3+}, the amplitude was increased due to OA by more than two-fold (Fig. 6B vs. 6E), whereas OM increased the amplitude by less than two-fold (Fig. 6A vs. 6E). These results indicate that OA in the presence of Fe^{3+} can also enhance the non-enzymatic production of hydroxyl radicals.

Ascorbic acid in combination with Fe (III)-complexes is considered to be a cause of non-enzymatic peroxidation. When ascorbic acid was added to the microsomes together with NADPH and Fe^{3+} (Fig. 7A) or together with NADPH, Fe^{3+} and OA (Fig. 7B), the amplitude of the EPR signal was reduced significantly compared with the samples without ascorbic acid. This effect might have been due to a reduction of the nitroxides by ascorbic acid, decreasing the amount of detectable spin adducts.

Studies with different combinations of NADPH and the antioxidant vitamin E (*dl*-α-tocopheryl acetate) demonstrated that the addition of vitamin E to the incubation mixture partially quenched the spectra relative to the controls (data not shown). The intensity of the signal with both vitamin E (12.5 mM final concentration) and NADPH was decreased by 15% compared with the sample where no vitamin E was added. In similar experiments [22] the EPR signal

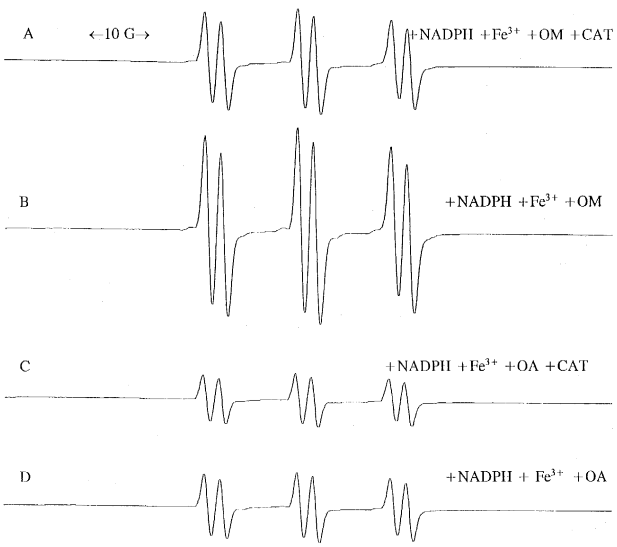


Fig. 5. EPR spectra of 4-POBN radical adducts following the incubation of rat liver microsomes with NADPH, Fe^{3+}, catalase, OA and OM-OA. Rat liver microsomes were isolated and incubated as described under Section 2. The spectra were recorded at 37°C immediately after adding 1 mM NADPH, 2.5 mM Fe^{3+}, 2% (v/v) ethanol and 100 mM 4-POBN, and were accumulated 8×. In addition, the samples contained: (A) 2.5 mM OM-OA, 0.1 mg catalase/ml; (B) 2.5 mM OM-OA; (C) 2.5 mM OA, 0.1 mg catalase/ml; (D) 2.5 mM OA.

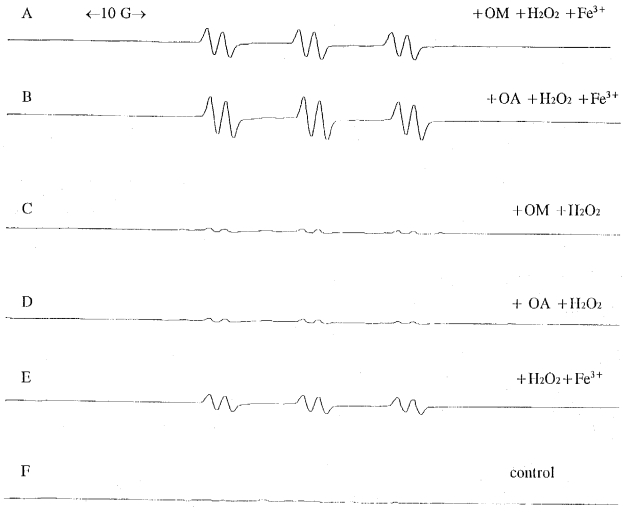


Fig. 6. EPR spectra of 4-POBN radical adducts following the incubation of microsomal storage solution with OA, OM, H_2O_2 and Fe^{3+}. The spectra were recorded at 37°C immediately after adding 2% (v/v) ethanol and 100 mM 4-POBN to the microsomal storage solution (0.1 M K_2HPO_4, pH 7.5), and were accumulated 8×. In addition, the samples contained: (A) 2.5 mM OM, 200 μM H_2O_2, 2.5 mM Fe^{3+}; (B) 2.5 mM OA, 200 μM H_2O_2, 2.5 mM Fe^{3+}; (C) 2.5 mM OM, 200 μM H_2O_2; (D) 2.5 mM OA, 200 μM H_2O_2; (E) 200 μM H_2O_2, 2.5 mM Fe^{3+}; (F) control.

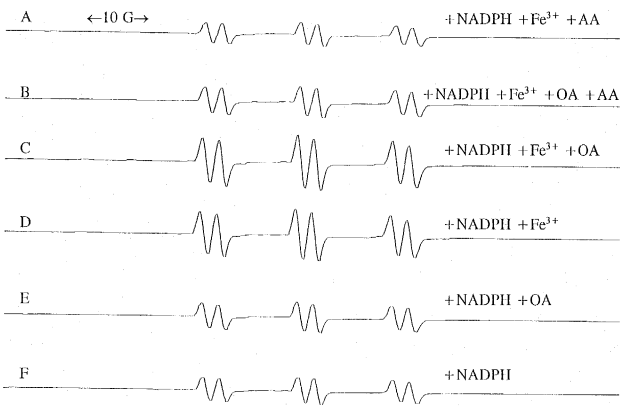


Fig. 7. EPR spectra of 4-POBN radical adducts following the incubation of rat liver microsomes with NADPH, OA, Fe^{3+} and ascorbic acid. Rat liver microsomes were isolated and incubated as described under Section 2. The spectra were recorded at 37°C immediately after adding 1 mM NADPH, 2% (v/v) ethanol and 100 mM 4-POBN, and were accumulated 8×. In addition, the samples contained: (A) 1 mM Fe^{3+}, 10 mM ascorbic acid; (B)1 mM Fe^{3+}, 2.5 mM OA, 10 mM ascorbic acid; (C) 1 mM Fe^{3+}, 2.5 mM OA; (D) 1 mM Fe^{3+}; (E) 2.5 mM OA; (F) control.

was completely inhibited following the addition of 500 mM *dl*-α-tocopheryl acetate to lung microsomes. Due to the relatively low water solubility of tocopheryl acetate in our system, only 12.5 mM of the antioxidant could be added to the incubation mixture without increasing the amount of ethanol added. That may explain the incomplete quenching effects in the present study.

An EPR signal was not observed in this study in the mitochondria and hepatocyte samples from rats treated with different agents (OA analogs, NADPH, Fe^{3+}, digitonin, Ca^{2+}, Ca ionophore A23187, and an uncoupler of oxidative phosphorylation carbonyl cyanide *m*-chlorophenylhydrazone-CCCP). Also, the utilization of a different spin trapping agent, α-phenyl-*N*-*tert*-butyl nitrone (PBN), resulted in no detectable radical adduct formation in these fractions. The concentration of the spin trapping agents in some samples was reduced from 100 to 50 and 25 mM, because according to Albano et al. [23], concentrations of PBN higher than 35 mM are not suitable for use with isolated hepatocytes due to possible interactions of PBN with the cytochrome-*P*-450 system and its role as a free radical scavenger. However, no detectable amounts of radical adducts were formed for all PBN concentrations, even at 100 mM.

4. Discussion

The spin adduct hyperfine splitting constants of the 6-line signals using 4-POBN as a spin trapping agent were measured to be $a_N = 15.4$ G and $a_H = 2.5$ G. This spin adduct has been identified as being 4-POBN-α-hydroxyethyl radical [18,19]. Finkelstein et al. [18] found hyperfine splitting constants for the 4-POBN α-hydroxyethyl adduct of $a_N = 15.56$ and $a_H = 2.59$ G as well as $a_N = 15.60$ G and $a_H = 2.65$ G under different experimental conditions. Augusto et al. [19] also reported hyperfine splitting constants for the same spin adduct of $a_N = 15.50$ and $a_H = 2.50$. According to Pou et al. [20], using 4-POBN in conjunction with ethanol is the most efficient spin trapping system for the detection of hydroxyl radicals in biological systems. Reinke et al. [24] also confirm these observations as their findings indicate that 4-POBN is the superior spin trapping agent for α-hy-

droxyethyl radicals. The initially generated hydroxyl radicals react with ethanol to yield α-hydroxyethyl radicals, which are then trapped by 4-POBN to form a very stable spin adduct. Pou et al. [20] concluded that the high efficiency of this procedure is attributable to the high sensitivity of this spin trapping system and the marked stability of the resulting spin trapped adduct, even in the presence of superoxide radicals which may be formed. We conclude that the same mechanism was responsible for the spin adducts in this work because both 4-POBN and ethanol (2% v/v) were added to all samples and essentially the same EPR spectra were obtained as those obtained by Pou et al. [20].

The results of this study confirm and extend previous data which has demonstrated that hydroxyl radicals are produced by microsomes in the presence of NADPH as the microsomal reductant and O_2, and without the addition of exogenous iron [6,24]. The results also suggest that OA at best only slightly enhanced the production of the hydroxyl radical over and above that obtained when microsomes were incubated in the presence of H_2O_2 and Fe^{3+}. The effect of OA also appeared to be non-enzymatic as a small but similar enhancement of the EPR signal was obtained when OA was added to buffer containing H_2O_2 and Fe^{3+} but no microsomes. In another study it was shown that catalase greatly reduced the EPR signals due to OA or OM-OA in combination with NADPH and Fe^{3+}, further suggesting that the mechanism of generation of the hydroxyl radicals in this study required a Fenton type of reaction involving H_2O_2. Hasinoff et al. [6] also showed that the Fe^{3+} complex of OA produced hydroxyl radicals in the presence of NADPH and NADPH cytochrome-*P*-450 reductase. The authors suggested that OA solubilizes Fe^{3+} by forming a complex, which accepts an electron to form Fe^{2+}-OA. This complex in turn reduces H_2O_2 yielding the hydroxyl radical. Hasinoff et al. [6] and Rahimtula et al. [1] attributed the enhanced free radical generation or lipid peroxidation, respectively, to the presence and reactivity of the phenolic hydroxyl group of OA. The authors concluded that there is a direct correlation between the toxicity of different analogs of OA, their dissociation constants (phenolic) and their iron chelating capacities. The results of the current study, as outlined above, indicate that OA, at best, only marginally enhances the production of free radicals and that the OA-Fe^{3+} complex is not a prerequisite for this reaction. This conclusion is further supported by the observation that OM-OA, a compound that does not have an ionizable phenolic group, greatly increased the EPR signal compared with that obtained with OA. In addition, this compound does not chelate iron but is toxic to mice [9]. In the EPR study of Hoehler et al. [7], OM-OA was also able to generate a relative strong EPR signal in bacteria. The authors, on the basis of results with their model system, concluded that there did not appear a close relationship between degree of ionization of the C-8 phenolate group and the production of the hydroxyl radical, or its overall toxicity to the microorganism. The current observations that OM-OA was the only OA analog to increase free radical generation significantly, that the OA-Fe^{3+} complex did not cause a significant increase of free radical generation when added together with NADPH, our previous results [7] and the results of Xiao et al. [9] therefore question the role of the phenolate in the generation of free radicals as the main mechanism of OA toxicity as hypothesized by other researchers. Taken together, the results further suggest that some other group within the OA molecule must be involved in the toxicity of OA.

In contrast to the results obtained with the microsomes, no EPR signal could be obtained when mitochondria or hepatocytes were used. In these systems the radical intermediates may have been produced at a concentration too low to be detected or the radical intermediate may not have been formed as a free species and therefore would not have been spin trapped. Poyer et al. [25] studied the formation of free radicals during the metabolism of halothane in four liver subcellular fractions: nuclei plus plasma membranes, mitochondria, microsomes, and the post microsomal supernatant fraction. The authors found that the microsomal fraction consistently showed an EPR signal and that it contained the major portion of the total spin trapped radicals present in the liver homogenate and the subfractions thereof. The current results, nevertheless, suggest that over a short period of time, OA does not produce a detectable EPR signal when added to liver cells or mitochondria. OA is known to have a high binding affinity for plasma albumin, which retards its elimination in vivo, by limiting the transfer of OA from the bloodstream to

the hepatic and renal cells [26]. As the medium used for the rat hepatocytes contained of 10% calf serum and 2% serum albumin, binding of OA to these proteins might have prevented cellular uptake of OA and therefore the generation of free radicals. Also, Meisner [27] found that OA has a poor affinity for isolated cell membranes and that the uptake of OA by mitochondria is an energy-utilizing process, resulting in a depletion of intra mitochondrial ATP. Further research is required to determine if the failure to produce an EPR signal in hepatocytes or mitochondria that have been exposed to OA and its analogs is attributable to a failure to take up the toxins, to a failure to induce free radical production, either directly or indirectly, or to some other cause.

In model studies using bacteria (*Bacillus brevis*) and OA analogs in EPR experiments, Hoehler et al. [7] recently found that OA and most of its analogs enhanced free radical generation. The EPR signals could be considerably enhanced by addition of Ca^{2+}, a Ca ionophore and CCCP (an uncoupler of oxidative phosphorylation). In the bacteria there was a close relationship between the actual uptake of the different OA analogs and the generated EPR signal. The more hydrophilic OA analogs OB, Oα and OP-OA, which were not taken up by the bacteria, did not result in a generation of detectable amounts of radical adducts in the bacterial system. In the present study, OA and its analogs might not have been taken up by the hepatocytes or the mitochondria in the aqueous solutions in contrast to the in vivo situation, where OA has been shown to be metabolized in liver microsomes from pigs, rats and humans to produce the hydroxylated derivatives (4*R*)- and (4*S*)-4-hydroxyochratoxin A [28–31]. OA metabolism has also been shown to be induced by sodium phenobarbital [28,30,31], and recently, Omar et al. [32] showed that OA is metabolized in the rat mainly by cytochrome P450 isoforms IA1/IA2, IIB1 and IIIA1/IIIA2. These authors also found that OA-induced lipid peroxidation lead to cytochrome P450 destruction, whereas a decreased lipid peroxidation and consequently lesser destruction of cytochrome P450 lead to increased OA metabolism. More evidence for the involvement of oxidative metabolism in the toxicity of OA is given by the finding that the genotoxicity of OA involves a glutathione conjugation reaction in bacteria suggesting the formation of a cytotoxic thiol-containing derivative [3]. Based on their results, Hoehler et al. [7] concluded that OA appears to produce many of the same effects as *tert*-butyl hydroperoxide and other prooxidants in the cell. Their results suggested that OA increases the permeability of the cell to Ca^{2+} and that both the enhanced cellular concentration of Ca^{2+} and the presence of the prooxidant OA uncouples oxidative phosphorylation resulting in an increased leakage of electrons from the respiratory chain producing $O_2^{\cdot-}$ and hence H_2O_2. A lack of an adequate supply of NAD(P)H and GSH to permit H_2O_2 consumption by the GSH dependent glutathione peroxidase and NAD(P)H dependent glutathione reductase together with an increased concentration of free iron within the cell stimulates the production of $^{\cdot}OH$ via the Fenton reaction due to mobilization of Fe^{2+} by Ca^{2+}. This results in further cell damage and may be one of the mechanisms that OA exerts its toxic effect. Consistent with this proposal is that a further metabolic inhibition by adding CCCP, an uncoupler of oxidative phosphorylation, to the bacteria resulted in a drastic enhanced EPR signal due to OA and Ca^{2+}. The results obtained in the current study support this hypothesis. The mechanism by which OA exerts its toxic effect, however, has not been established. Studies by Xiao et al. [9] have suggested that the toxicity of OA is associated with its isocoumarin moiety and that its lactone carbonyl group appears to be involved.

It is concluded that OA only slightly increases the magnitude of the EPR signal over and above that obtained when microsomes are incubated in the presence of NADPH and H_2O_2, that the signal can be produced non-enzymatically, that the hydroxyl radical is produced, that neither an OA-Fe^{3+} complex nor the dissociation of the phenolate group are essential for the production of the hydroxyl radical and that the effects of OA on the intact cell may be caused by the production of hydroxyl radicals indirectly rather than directly.

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